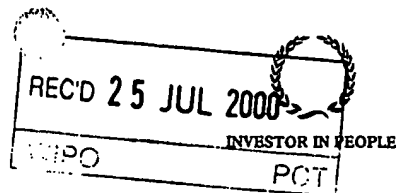




G800
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#2

**PRIORITY
DOCUMENT**

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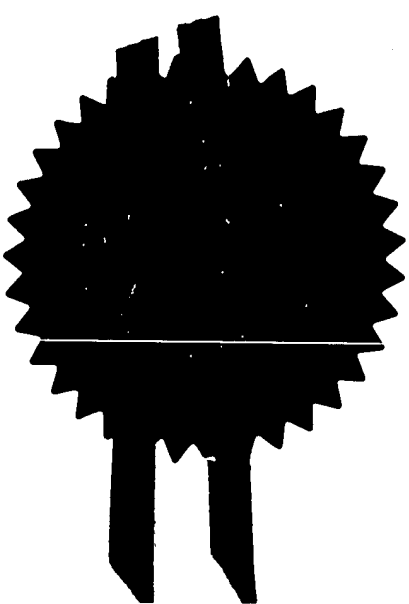
4

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

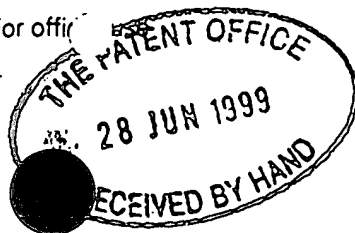
Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed *Andrews*

Dated 11 July 2000

For office


 29 JUN 99 E458042-1 003312
 P01/7700 0.00 - 9915069.0

Your reference 230P80900

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Notes

Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071-438 4700).

Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.

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After an application for a Patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977 and will inform the applicant if such prohibition or restriction is necessary. Applicants resident in the United Kingdom are also reminded that under Section 23, applications may not be filed abroad without written permission unless an application has been filed not less than 6 weeks previously in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction revoked.

**The
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Request for grant of a Patent

Form 1/77

Patents Act 1977

1 Title of invention

- 1 Please give the title of the invention **NEW INDOLOCARBAZOLE ALKALOIDS FROM A MARINE ACTINOMYCETE**

2 Applicant's details☐ **First or only applicant**

- 2a If you are applying as a corporate body please give:

Corporate name

Instituto Biomar S.A.

 Country (and State
of incorporation, if
appropriate)

Spain

- 2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

- 2c In all cases, please give the following details:

Address

 Poligono Industrial, edificio CEI
 Mod. 2.02
 24231-Onzonilla (LEON)
 Spain

 UK postcode
 (if applicable)

Country

 ADP number
 (if known)

7262868002

2a, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper.

☐ **Second applicant (if any)**

2d If you are applying as a corporate body please give:

Corporate name

Country (and State
of incorporation, if
appropriate)

2e If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2f In all cases, please give the following details:

Address

UK postcode
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Country

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Ⓜ An address for service in the United Kingdom must be supplied

Please mark correct box

Ⓜ Address for service details

3a Have you appointed an agent to deal with your application?

Yes ☒

No ☐ → go to 3b

↓
please give details below

Agent's name

Marks & Clerk

Agent's address

57-60 Lincoln's Inn Fields.
LONDON
WC2A 3LS.

Postcode

Agent's ADP
number

18001

3b: If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

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(if known)

Daytime telephone
number (if available)

Reference number

4 Agent's or
applicant's reference
number (if applicable)

230P80900

⑤ Claiming an earlier application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Yes ☐

No ☐ $x \Rightarrow$ *go to 6*

please give details below

number of earlier application or patent number

filing date

(day month year)

☐ and the Section of the Patents Act 1977 under which you are claiming:

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

⑥ Declaration of priority

6 If you are declaring priority from previous application(s), please give:

Country of filing

Priority application number
(if known)

Filing date
(day, month, year)

⑥ *If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number.*

Please give the date in all number format, for example, 31/05/90 for 31 May 1990.

- 7 answer must be 'No' if:
- any applicant is not an inventor
 - there is an inventor who is not an applicant, or
 - any applicant is a corporate body.

8 Please supply duplicates of claim(s), abstract, description and drawing(s).

Please mark correct box(es)

- 9 You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request.

Please sign here ➡

A completed fee sheet should preferably accompany the fee.

7 Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint inventors?

Please mark correct box

Yes ☐

No ☒

➡ A Statement of Inventorship on Patents Form 7/77 will need to be filed (see Rule 15).

8 Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77

Claim(s)

Description

 10

Abstract

Drawing(s)

 7

(Drawings)

8b Which of the following documents also accompanies the application?

Priority documents (please state how many)

Translation(s) of Priority documents (please state how many)

Patents Form 7/77 – Statement of Inventorship and Right to Grant
(please state how many)

Patents Form 9/77 – Preliminary Examination/Search

Patents Form 10/77 – Request for Substantive Examination

9 Request

I/We request the grant of a patent on the basis of this application.

Signed Marks & Clerk

Date 28-6-99
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New Indolocarbazole Alkaloids from a Marine Actinomycete

FIELD OF THE INVENTION

New indolocarbazole alkaloids have been isolated from the culture broth of a staurosporine-producing actinomycete (CLCO-002). Their production by aerobic fermentation under controlled conditions of the strain, and the isolation and purification of compounds are described herein. The compounds and the fermentation broth demonstrate significant activity against several cancer cell lines.

BACKGROUND OF THE INVENTION

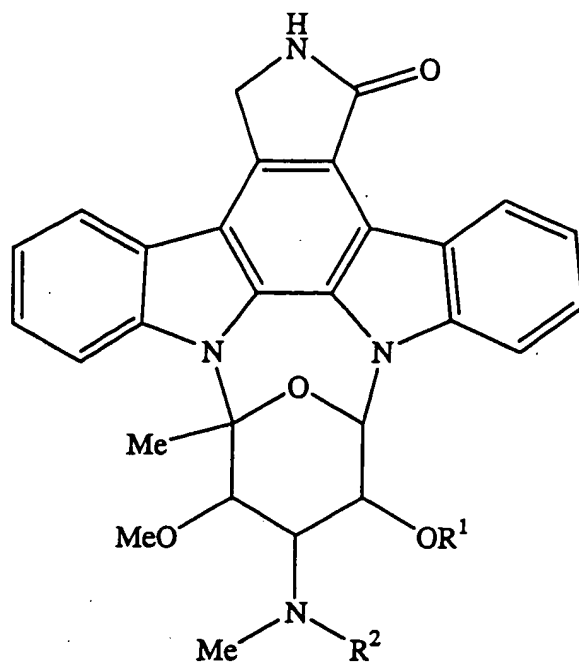
The isoenzyme family of protein kinase C (PKC) plays a key role in signal transduction and cellular regulation (Y. Nishizuka, 1988). From the observation that the tumor promoting phorbol esters are able to stimulate PKC activity (Y. Nishizuka, 1984), it was concluded that inhibitors of this enzyme could be useful for cancer chemotherapy. PKC inhibitors have been extensively investigated as potential drugs for the treatment of cancer. Accordingly, a goal of the present invention is to provide new antitumor agents; these compounds are alkaloids with significant activity against several cancer cell lines.

Yet another objective of this invention is to provide pharmaceutical compositions for administering to a patient in need of treatment using the active compounds described herein.

Microbial products are interesting because their industrial production is well established at present times. Therefore, another objective of this invention is directed to the production of the active compounds and to their isolation and purification from the resulting fermentation broth.

SUMMARY OF THE INVENTION

This invention provides compounds with the proposed indolocarbazole formula (1).

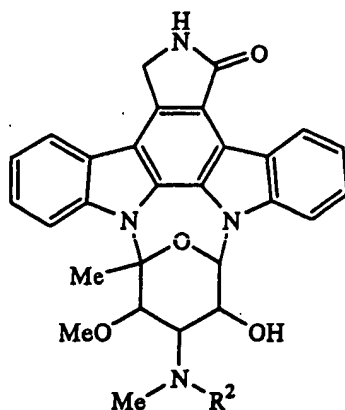


(1)

wherein R^1 and R^2 are independently selected from the group consisting of hydrogen atoms, lower alkyl groups and lower alkoxy groups.

In the definitions of the groups in formula (1), the lower alkyl and the lower alkyl moiety of the lower alkoxy mean a straight-chain or branched alkyl group having 1 to 6 carbon atoms such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl and hexyl.

More particularly, the present invention relates to IB-97224 and IB-97225, with structural formula:



IB-97224 ($R^2=Me$)

IB-97225 ($R^2=H$)

In this invention the process of obtaining IB-97224 and IB-97225 is also described, and the preferred process comprises cultivating a strain of a microorganism capable of producing IB-97224 and IB-97225 in an aqueous nutrient medium with assimilable carbon and nitrogen sources and salts, under controlled submerged aerobic conditions. The compounds IB-97224 and IB-97225 are recovered and purified from the cultured broth.

The preferred culture is strain CLCO-002, and its chemical, biochemical and morphological characters show that it belongs to the Actinomycetales group.

As described above, the compounds IB-97224 and IB-97225 have been found to have good activity against murine and human tumor cell lines, including P-388D₁, HT-29, A-549 and SK-MEL-28.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an ultraviolet spectrum (UV) of purified IB-97224 (t_R = 3.92 min) and IB-97225 (t_R = 3.29 min);

Figures 2A and 2B are the infrared spectra (IR) of purified IB-97224 and IB-97225 respectively;

Figures 3A and 3B are the proton NMR (1H) spectra of purified IB-97224 and IB-97225 respectively; and

Figures 4A and 4B are the mass spectra of purified IB-97224 and IB-97225 respectively.

DETAILED DESCRIPTION OF THE INVENTION

The Producing Organism

The microorganism utilized for the production of these new compounds is preferably the actinomycete strain CLCO-002, a culture of which has been deposited in the Colección Española de Cultivos Tipo at the University of Valencia, Spain under the accession number CECT-3347. This deposit has been made under the provisions of the Budapest Treaty and all restrictions on the availability thereof to the public will be irrevocably maintained upon the granting of a patent on this application.

The organism was isolated from an unidentified marine sponge collected in Canary Islands waters.

All cultures were incubated at 27°C and records of results were made weekly up to 21 days.

A description of the organism is as follows:

Morphology

The culture media utilized for this study were, ISP media No 2, 4, 5 and 6 (Shirling and Gottlieb, 1966), ATCC medium No 172 (American Type Culture Collection Catalog, 1989), Czapek Agar (Atlas, 1993), Bennet Agar (Atlas, 1993), 1.5% Water Agar (Luedemann). All media were supplemented with 50% artificial sea water. After 21 days at 28°C growth was studied. Several shades of orange were observed. No aerial mycelium was formed. Substrate mycelium was branched. No soluble pigment was observed.

Physiological characteristics

For carbon and nitrogen utilization studies ISP-9 was used (Shirling & Gottlieb, 1966). Due to low growth rate of CLCO-002 under defined media, the carbon and nitrogen utilization tests showed residual growth so no clear results could be obtained. NaCl resistance was determined by using ATCC's 172 medium containing increasing concentrations of NaCl. The optimal concentration of salt was 1%. No growth was observed with 7% salt.

Cell chemical composition

Aminoacids:

Diaminopimelic acid was determined by the method of Hasegawa et al.(1983). The *meso*-2,6-Diaminopimelic acid isomer was present in the whole cell hydrolysate of strain CLCO-002.

Fatty acids:

FAMES were determined by the method of Van der Auwera et al. (1986). The FAME composition as well as comparison with other similar strains is described in Table 1.

While the deposited organism is clearly preferred, the present invention is not restricted or limited to this particular strain or organisms. It is the intention of the present inventors to include any other producing organisms, strains or mutants within the scope of this invention.

TABLE 1

FAME composition of strain CLCO-002 and other actinomycetes strains. Composition is given as percentage of total fatty acids content.

	13:0	i-14:0	14:0	i-15:0	15:0	i-16:1	i-16:0	16:1	16:0	i-17:1	i-17:0	17:1	17:0	i-18:1	i-18:0	18:1	18:0		
CLCO-002	<1	<1	<1	16.91	3.94	6.71	<1	31.83	<1	<1	3.73	<1	<1	24.33	3.31	<1	<1	4.13	<1
STALBUS	<1	6.52	<1	9.88	22.92	<1	5.50	25.29	<1	3.75	1.28	3.38	8.60	<1	<1	<1	1.09	<1	<1
SPAMETH	1.21	10.34	<1	1.86	<1	4.30	<1	15.51	5.63	8.62	1.08	<1	<1	24.02	9.43	7.11	<1	4.60	1.04
SPVIRIDO	<1	4.04	1.10	18.94	2.71	4.89	<1	26.44	<1	4.43	<1	2.60	1.58	11.36	8.58	7.48	<1	<1	1.16
AMCITRE	<1	<1	3.18	<1	<1	1.03	<1	6.37	12.62	40	<1	<1	<1	<1	1.16	<1	<1	14.25	2.82
APBRAZIL	<1	3.15	<1	15.46	18.91	2.76	<1	19.07	2.15	1.79	<1	2.39	9.64	11.18	2.82	<1	<1	3.38	1.06
AMPDIGIT	<1	11.57	<1	11.21	9.96	<1	2.87	34.23	<1	1.08	<1	1.28	5.08	4.39	1.64	<1	1.76	7.60	1.54
AMYORIE	<1	3.40	2.37	19.94	4.66	1.17	<1	11.85	5.59	18.41	<1	2.99	4.44	3.09	2.73	<1	<1	6.21	3.04
MNCHALC	<1	1.68	<1	8.91	2.29	1.53	1.15	38.23	<1	1.88	1.49	2.32	2.25	5.43	6.95	14.58	1.31	1.28	2.68
MNECHCA	<1	1.17	<1	6.97	1.24	2.81	<1	30.88	<1	2.29	1.63	4.11	1.68	12.15	4.90	7.23	<1	10.05	1.69
MNFUSCA	<1	<1	<1	26.56	6.53	<1	<1	8.58	<1	<1	7.30	11.89	13.25	2.90	3.37	3.59	<1	2.33	1.94
SACCAER	<1	3.06	1.35	14.41	8.62	1.04	5.68	20.07	13.84	6.16	4.55	2.20	5.31	2.02	<1	<1	<1	<1	1.43
NOAFRI	1.51	5.43	3.35	4.62	<1	7.46	3.09	22.18	2.69	5.15	2.35	<1	<1	8.15	4.75	17.03	<1	<1	1.23
MTSALMO	<1	1.12	1.28	6.75	<1	7.83	7.53	21.58	1.21	1.97	1.01	<1	1.07	11.58	5.53	17.34	<1	<1	<1
MTRUBRA	<1	1.40	1.38	4.12	<1	3.41	7.27	25.00	2.63	3.89	2.17	1.08	<1	6.84	4.97	15.44	1.25	<1	1.61
MTROSEO	2.03	3.65	5.14	3.86	<1	9.03	3.02	12.31	3.46	6.95	1.17	<1	<1	13.51	4.46	18.67	<1	1.77	<1
AMROSEO	<1	2.19	1.24	6.73	1.09	6.94	1.43	22.21	2.21	3.61	2.74	1.03	<1	10.97	4.33	17.84	<1	<1	<1
MTFERRU	1.03	1.91	1.19	1.94	<1	6.43	4.12	21.50	2.32	2.34	<1	<1	<1	23.51	5.71	12.15	1.27	1.43	<1

CLCO-002 = strain CLCO-002; AMCITRE = *Actinomadura citrea* DSM 43461; AMPDIGIT = *Ampullariella digitata* ATCC 15349; AMROSEO = *Actinomadura roseoviolacea* DSM 43144; AMYORIE = *Amycolatopsis orientalis* DSM 40040; APBRAZIL = *Actinoplanes braziliensis* ATCC 25844; MNCHALC = *Micromonospora chalcone* ATCC 31395; MNECHCA = *Micromonospora echinospora calichinensis* NRRL 15839; MNFUSCA = *Micromonospora fusca* NRRL B-3298; MTFERRU = *Microtetraspora ferruginea* DSM 43553; MTROSEO = *Microtetraspora roseola* ATCC 33579; MTRUBRA = *Microtetraspora rubra* ATCC 27031; MTSALMO = *Microtetraspora salmonea* ATCC 33580; NOAFRI = *Nocardiopsis africana* DSM 43748; SACCAER = *Saccharothrix aerocolonigenes* NRRL B-3298; SPAMETH = *Streptosporangium amethystogenes* DSM 43179; SPVIRIDO = *Streptosporangium viridogriseum* ATCC 25242; STALBUS = *Streptomyces albus* DSM 40313

Fermentation

Strain CLCO-002, when cultured under controlled conditions in a suitable medium produces the compounds IB-97224 and IB-97225. This strain is grown in an aqueous nutrient medium, under aerobic and mesophilic conditions, preferably between 22°C and 35°C at a pH ranging between 6.0 and 8.0. A wide variety of liquid culture media can be utilized for the cultivation of the organism, useful media are those that include an assimilable carbon source, such as starch, dextrin, sugar molasses, glycerol, glucose and the like, an assimilable nitrogen source such as proteins, protein hydrolysates, defatted meals, corn steep, and the like, and useful inorganic anions and cations such as sodium, magnesium, potassium, ammonium, sulphate, chloride, phosphate, carbonate, and the like. Trace elements may be added also. Aeration is preferably achieved by supplying air to the fermentation medium. Agitation is provided by a mechanical impeller. Conventional fermentation tanks have been found to be well suited for carrying out the cultivation of this organism. The addition of nutrients and pH control as well as antifoaming agents during the various stages of fermentation may be needed for increasing production and avoid foaming.

The required steps needed for production of these compounds by the preferred organism are:

Start with frozen or lyophilised mycelium. Obtain mycelial mass culturing the initial cells in shake flasks with a culture medium containing some of the ingredients described above at mesophilic temperatures and in aerobic conditions, this step may be repeated several times, as needed, and the material collected will be used as an inoculum to seed one or several fermentation tanks with any appropriate culture medium, if desired these tanks can be utilised also as inoculum, and this step can be repeated several times when needed, or they can serve as the production stage, depending on the broth volume needed. The production stage can last from very few days to more than one week, depending on strain, inoculum stages, temperature and other conditions. Once the fermentation has reached its maximum yield can be harvested for the isolation of the new compounds.

Production medium may be different than that used as inoculum. In Table 2 typical media are described that can be used for inoculum and production of these new compounds:

TABLE 2

<u>Inoculum medium (g/litre)</u>		<u>Production medium (g/litre)</u>	
Dextrose	5	Dextrose	5
Starch	20	Dextrin	20
Beef extract	3	Soybean meal	3
Yeast extract	5	Yeast extract	5
Peptone	5	Peptone	1
CaCO ₃	4	CaCO ₃	4
NaCl	4	NaCl	5

Na ₂ SO ₄	1	Na ₂ SO ₄	2.5
KCl	0.5	KCl	0.5
MgCl ₂	2	MgCl ₂	0.5
K ₂ HPO ₄	0.5	K ₂ HPO ₄	0.5
		(NH ₄) ₂ SO ₄	0.5
Tap water to 1,000 ml			

Production of these compounds can be monitored by whole broth assay against A-549 or any other sensitive cell or by HPLC or any other method with enough sensitivity.

Isolation of IB-97224 and IB-97225

Alkaloids IB-97224 and IB-97225 can be isolated from the mycelia cake by extraction with a suitable mixture of solvent such as CHCl₃:CH₃OH:H₂O. The activity is concentrated in the lower layer. The extracts from two repeated extraction can be combined and evaporated to dryness *in vacuo*.

Separation and purification of IB-97224 and IB-97225 from the crude active extract can be performed by the use of the proper combination of conventional chromatographic techniques.

Fractionation can be guided by the antitumor activity of fractions, or by TLC visualized with vanillin in conc. H₂SO₄, or analytical HPLC with photodiode-array detector. HPLC analysis are performed at room temperature (Waters RCM 8x10, 8C18 10μm cartridge) using as mobile phase acetonitrile-sodium hydrogenphosphate 0.025M pH=3 (75:25) and a flow rate of 2 ml/min. and plotted at 290 nm. Compounds of interest showed retention times of 3.92 and 3.29 minutes to IB-97224 and IB-97225 respectively.

On the basis of detailed analysis of their various spectral characteristics, the pure compounds can be identified as IB-97224 and IB-97225 (see data reproduced in FIGS. 1 to 4B).

The U.V. spectra show absorption at about 206, 242, 291, 320, 334, 354 and 370nm in acetonitrile-sodium hydrogenphosphate 0.025M pH=3 (75:25).

The infrared absorption spectra in KBr are shown in FIGS. 2A and 2B of the accompanying drawings.

The ¹H spectra are reported in FIGS. 3A and 3B.

The mass spectra are shown in FIGS. 4A and 4B which displayed (M+H)⁺ ions at m/z 497.2 and 483.2 for IB-97224 and IB-97225 respectively.

Biological activity

The antitumor activities of IB-97224 and IB-97225 have been determined *in vitro* in cell cultures of mouse leukemia P-388D₁, human lung carcinoma A-549, human colon carcinoma HT-29 and human melanoma SK-MEL-28. The procedure was carried out using the methodology described by Bergeron, et al. (1984), and by Schroeder, et al. (1981).

IB-97224 and IB-97225 exhibit antitumor activities against several mammalian tumors cells. Also provides a method of treating any mammal affected by a malignant tumor sensitive to IB-97224 and IB-97225, which comprises administering to the affected individual a therapeutically effective amount of IB-97224 and IB-97225 or a pharmaceutical composition thereof.

The present invention also relates to pharmaceutical preparations which contain as active ingredient compounds IB-97224 or IB-97225, or a pharmaceutical acceptable acid addition salt thereof, as well as the processes for its preparation.

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable composition for oral, topical or parenteral administration, and they may contain the pure compounds or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

The correct dosage of a pharmaceutical composition of IB-97224 or IB-97225 will vary according to the particular formulation, the mode of application, and the particular *situs*, host and bacteria or tumor being treated. Others factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken in account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

The present invention will be further illustrated with reference to the following examples which aid in the understanding of the present invention, but which are not to be construed as limitations thereof. All percentages reported herein, unless otherwise specified, are presented by weight. All temperatures are expressed in degrees Celsius. All incubations are carried out at 28 °C and flasks are shaken in an orbital shaker. All media and recipients are sterile and all culture processes aseptic.

EXAMPLE 1

Stock Culture: Whole broth of a pure culture of strain CLCO-002 is preserved frozen in 20% glycerol.

Inoculum: A frozen culture or a well grown slant culture (5% vol.) is used to seed 100 ml of seed medium described previously contained in a 250 cc shake flask. The flask is incubated during 48 hr. 500 ml of the same medium in 2 L erlenmeyer flask are seeded with 10% of the first stage inoculum. The flask is incubated during 48 h.

Fermentation: With 2.5 L of second stage inoculum seed 50 L of production medium already described in a 75 L fermentation tank. The fermentation is carried out during 96 hours with 400 rpm agitation and an air flow of 0.5 V/V.M.

Monitor secondary metabolite production by assay of whole broth against A-549 or by HPLC.

Isolation: 10 L of whole harvested broth were filtrated to separate the biomass and other solids. The mycelial cake was extracted twice with a mixture solvent (2.4 l) of CHCl_3 : CH_3OH : H_2O (2:1:1), and the activity was concentrated in the lower layer. The organic solvent was concentrated and evaporated to dryness *in vacuo* to yield 3.2 g of crude extract. The extract was chromatographed on silica gel "vacuum flash" column. After washing with a mixture of n-hexane-ethyl acetate 1:1, the column was developed with an ethyl acetate-methanol gradient. The progress of the elution was checked for cytotoxicity against A-539 cells and monitored by TLC (chloroform-methanol 9:1) and analytical reverse phase HPLC-photodiode array. Further purification of active fractions (250 mg) was achieved by column chromatography on silica gel and the activity was eluted with chloroform-methanol 92:8 and 95:5. Each of these fractions were chromatographed on a column of C18 reversed phase and eluted with methanol-water 65:35 to give 12 mg of staurosporine, 4 mg of IB-97224, and 8 mg of IB-97225.

Biological activity: The antitumor cells employed have been P-388D₁ (suspension culture of a lymphoid neoplasm from DBA/2 mouse), A-549 (monolayer culture of a human macrocytic lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma), and SK-MEL-28 (monolayer culture of a human melanoma). P-388D₁ cells were seeded into 16 mm wells at 1×10^4 cells per well in 1 ml aliquots of MEM 5FCS containing the indicated concentration of drug. A separate set of cultures without drug were seeded as control of growth to ensure that cells remained in exponential phase of growth. All determinations were carried out duplicated. After three days of incubation at 37 °C in 10% CO₂ atmosphere with 98% humidity, the IC₅₀ was calculated by comparing the growth in wells with drug with the growth in control wells without the drug. A-549, HT-29, and SK-MEL-28 cells were seeded into 16 mm wells at 2×10^4 cells per well in 1 ml aliquots of MEM 10FCS containing the indicated concentration of drug. A separate set of cultures without drug were seeded as control of growth to ensure that cells remained in exponential phase of growth. All determinations were carried out duplicated. After three days of incubation at 37°C in 10% CO₂ atmosphere with 98% humidity, the well were stained with 0.1% Crystal Violet. The IC₅₀ was calculated by comparing the growth in wells with drug with the growth in control wells without the drug.

In Table 3 are presented the activity expressed as IC₅₀ (μM)

TABLE 3

Cell line	IC ₅₀ (μM)	
	IB-97224	IB-97225
P388D ₁	0.04	0.02
A-549	0.002	0.002
HT-29	0.004	0.004
SK-MEL-28	0.004	0.002

Cited References

The following references have been cited herein, and they are hereby incorporated herein by reference:

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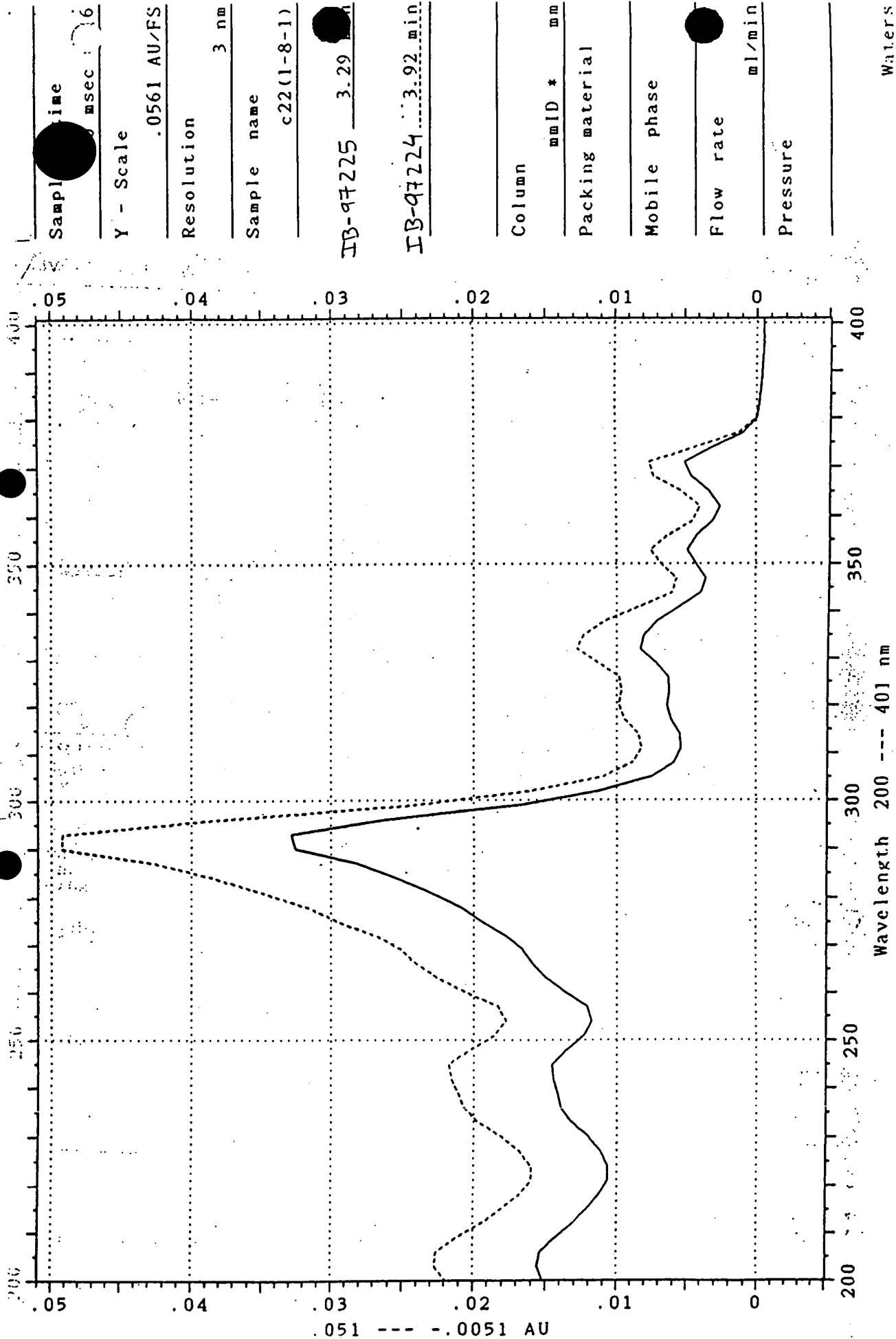


FIG. 1

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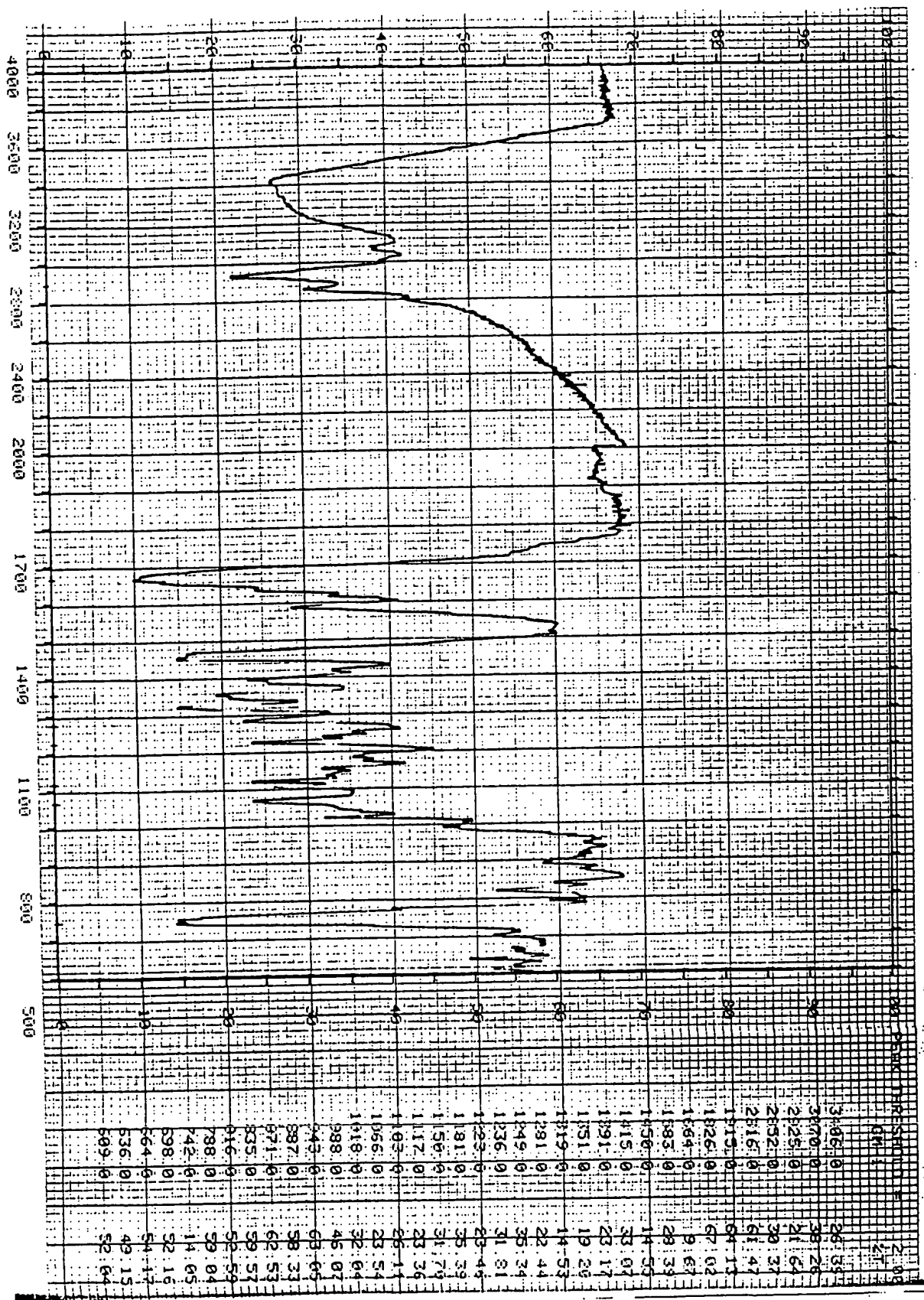


FIG. 2A

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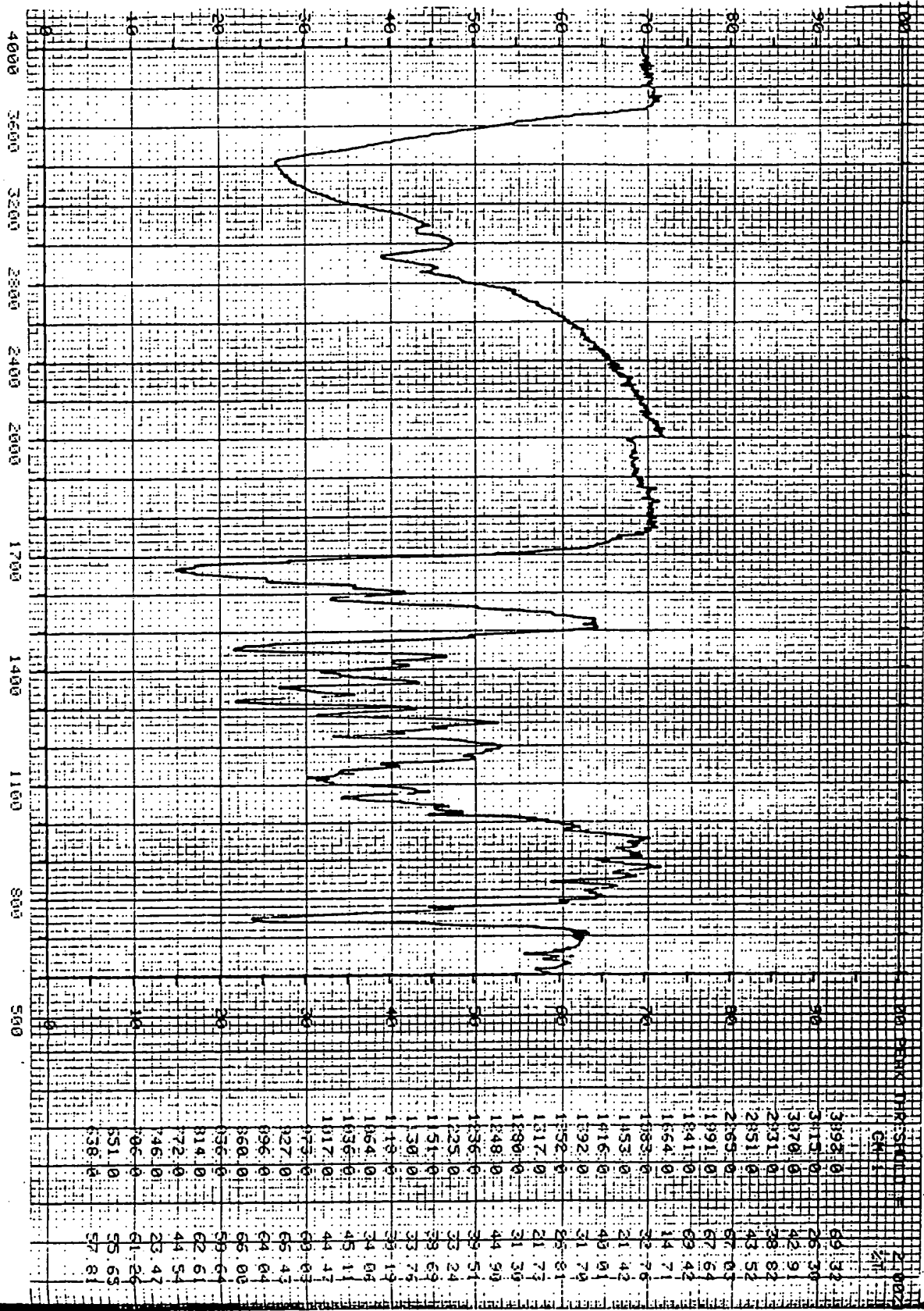


FIG. 2B

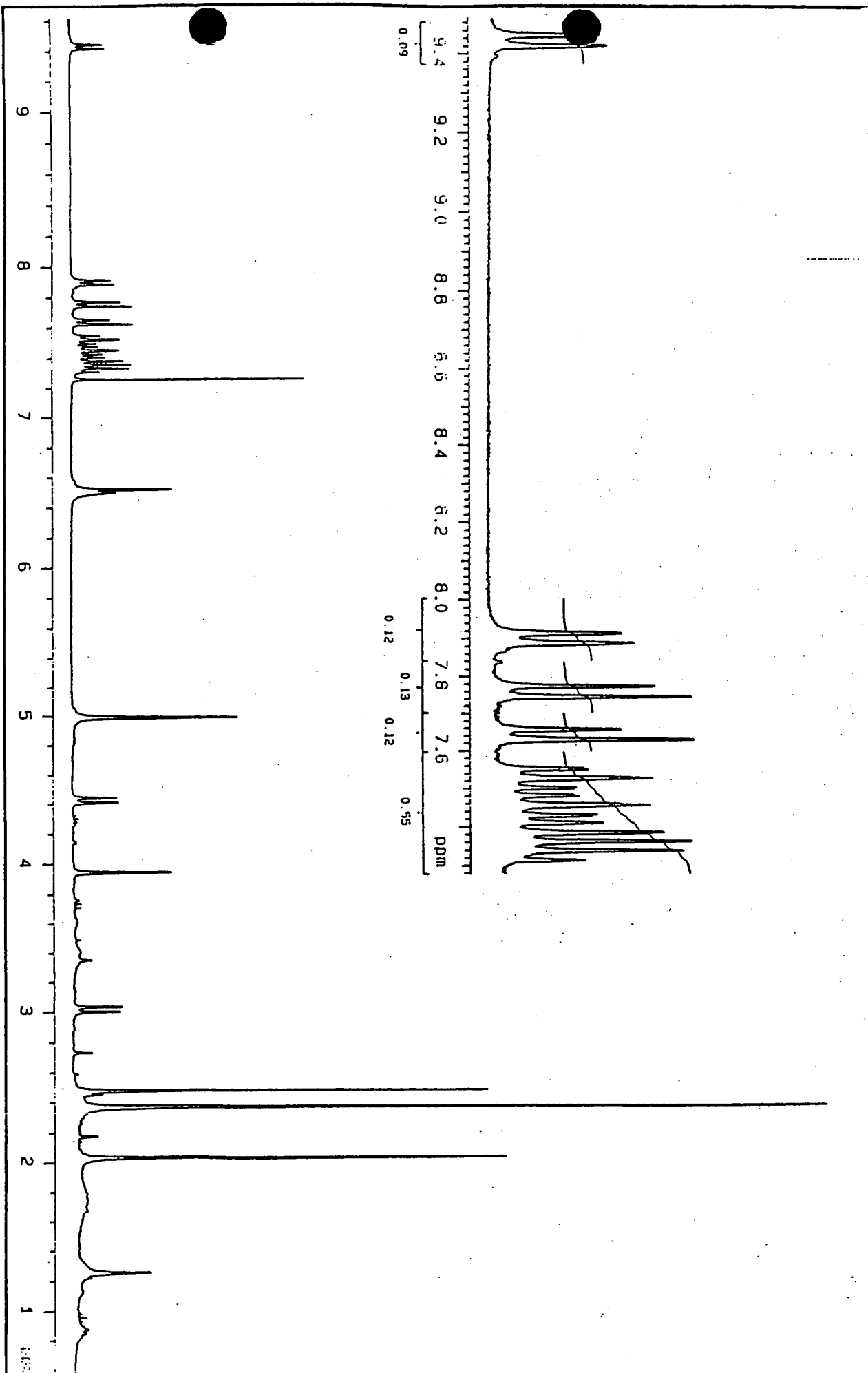


FIG. 3A

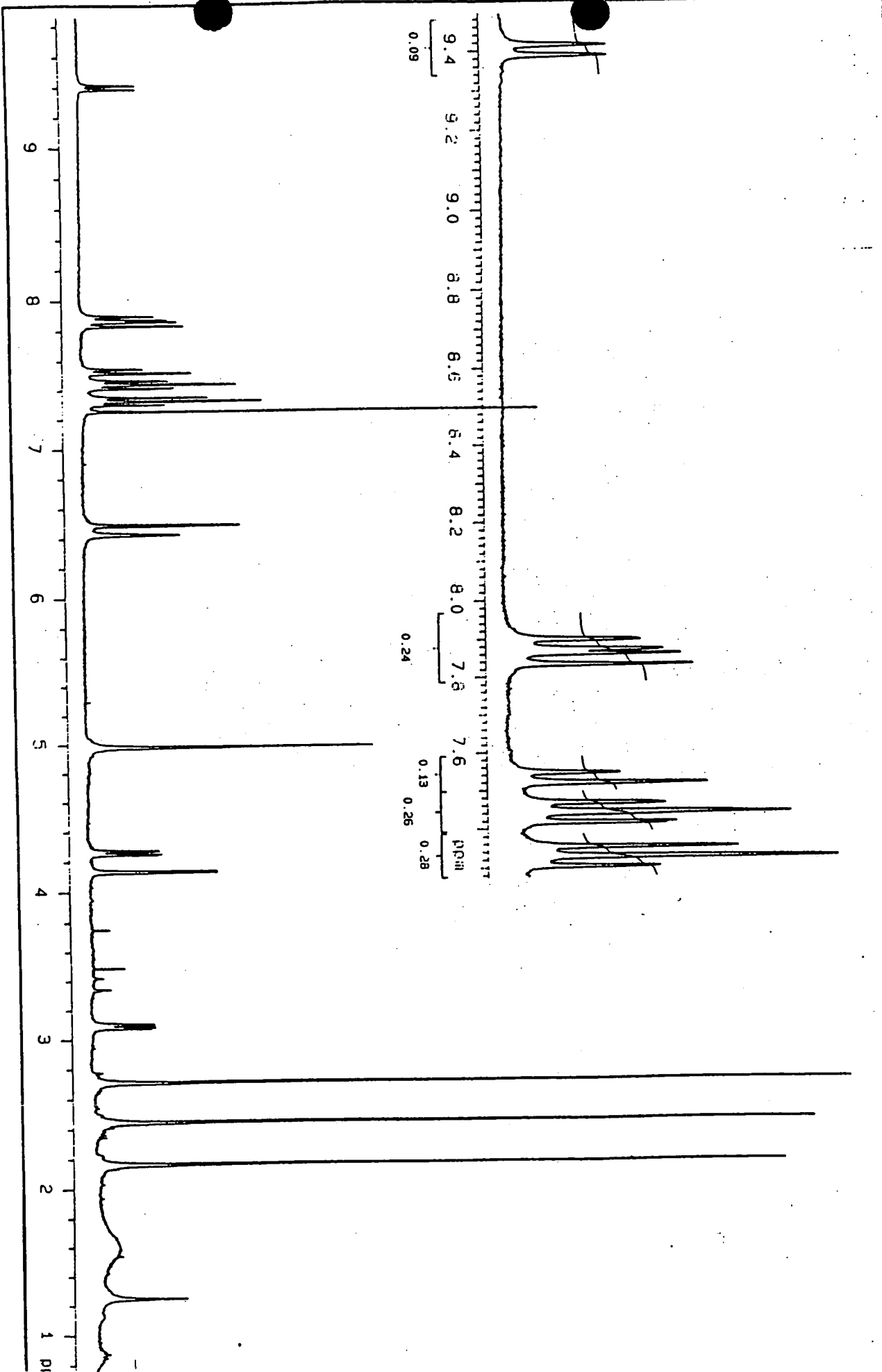


FIG. 3B

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 File Text: HUANG AL2-002-6/5 5 IN M.B.

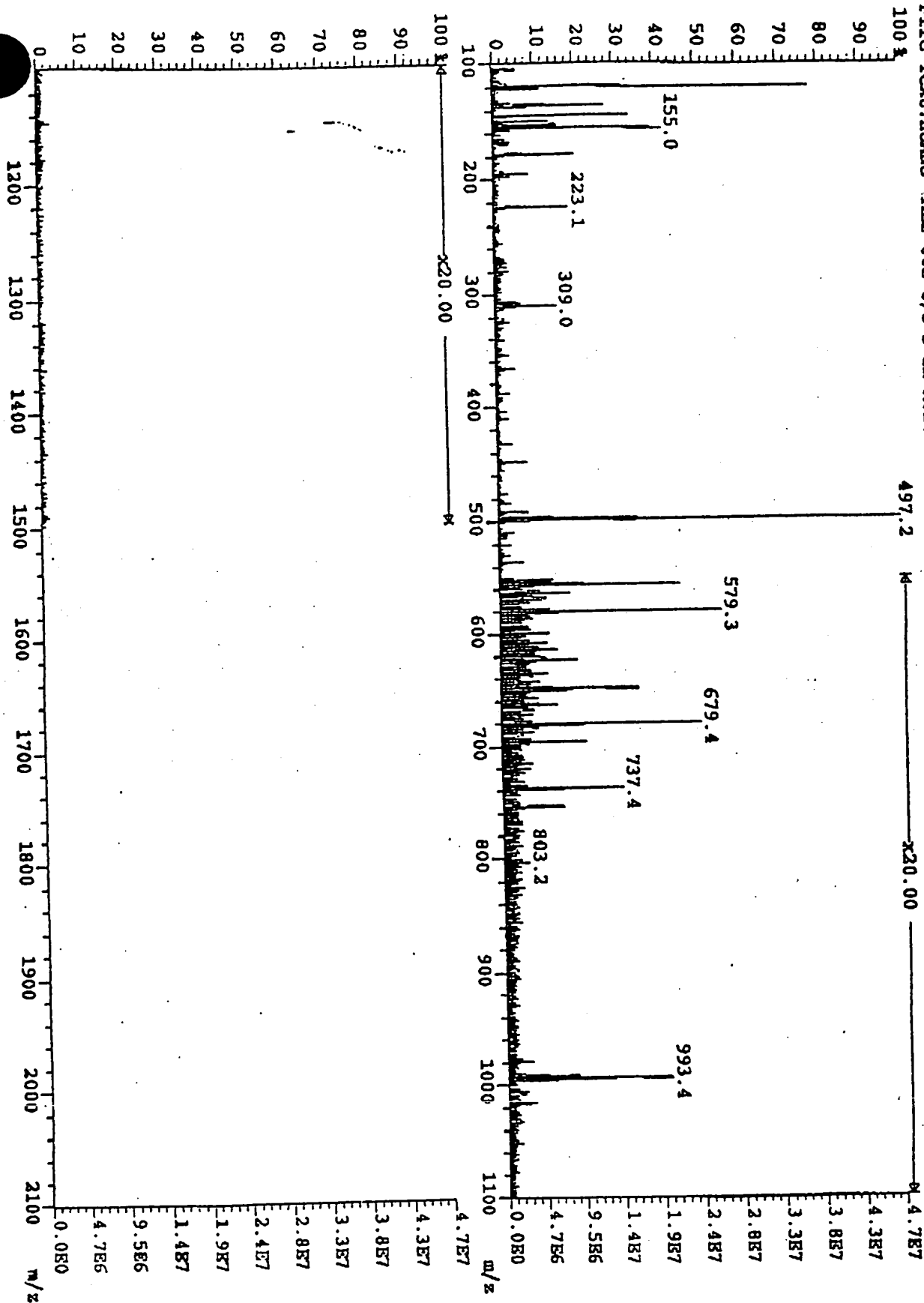


FIG. 4A

617

File:AL261046 Ident:2 Acq: 7-AUG-1997 11:48:34 +1:03 Cal:CSI080797
ZAB-SE4P FAB+ Magnet BPV:12.1V BpI:96823344 TIC:4304283648 Plags:HALT
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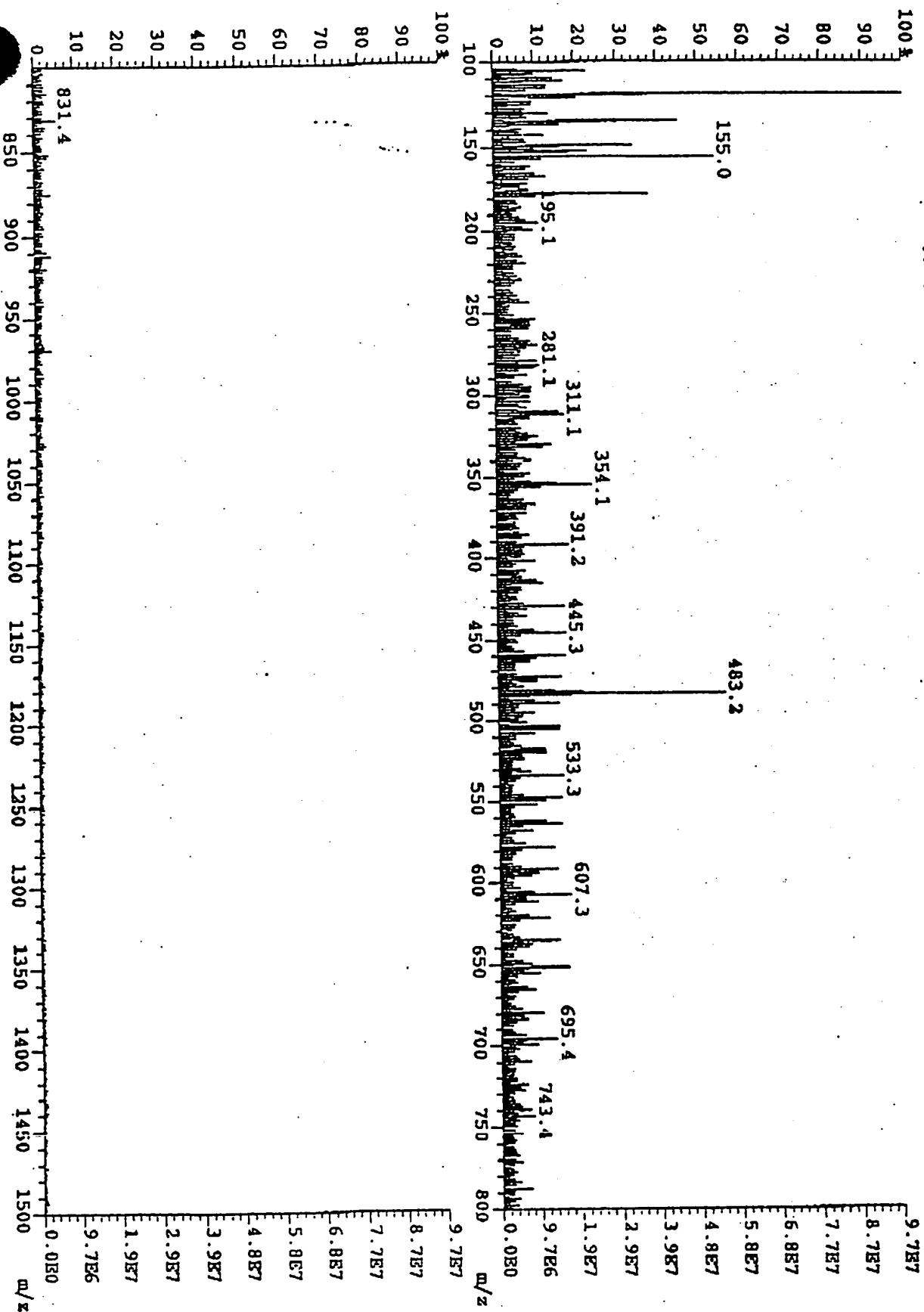


FIG. 4B